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SUPPLEMENTATION WITH FRUIT AND VEGETABLE EXTRACTS MAY DECREASE DNA DAMAGE IN THE PERIPHERAL LYMPHOCYTES OF AN ELDERLY POPULATION

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ABSTRACT

Fruit and vegetable consumption has been heralded for its ability to decrease the overall risk of developing cancer and other diseases. Mounting evidence supports the beneficial nature of antioxidants, carotenoids, and other phytonutrients found in fruits and vegetables. One proposed mechanism of antioxidant protection is the shielding of cellular DNA from oxidative damage and therefore mutations. This may be especially helpful in older populations. We tested the concept that a daily regimen of supplementation with fruit and vegetable extracts (JuicePlus™) would reduce the amount of DNA damage in the peripheral lymphocytes of the elderly. In a blind study, a group of twenty elderly volunteers (mean age= 68) were given supplements twice daily for 80 days with blood samples drawn before and after intervention. These samples were compared using the comet assay, a technique that quantifies DNA damage to individual nuclei. Each sample was tested in triplicate, and tail moment data was collected from over 200 comets per sample. Paired t-test analysis revealed a highly significant ($p<0.0001$) decrease in measured DNA damage between pre (13.24±2.77) and post (4.41±2.76) treatment tail moment. Screening of test subjects' personal data showed no apparent relationship between age, sex, or smoking. In this initial study, we conclude that a daily course of fruit and vegetable extract supplementation may reduce the level of DNA damage found in the peripheral lymphocytes of seniors.

Key Words: Antioxidants, Aging, Cancer, Comet assay, Prevention.

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INTRODUCTION

Fruits and vegetables are readily available in developed countries and are a natural source of nutrients, fiber, and potent antioxidants. Significant research has been done to uncover many beneficial effects of fruits and vegetables and the compounds responsible for the observed phenomena. Examples of these useful chemicals include vitamins C and E, carotenoids, and flavonoids (1,2,3). Each fruit and vegetable has unique combinations and concentrations of these compounds, therefore a broad sampling of produce in an individual's diet provides the greatest advantage.

Not all people follow a diet that includes high intake of fruits and vegetables. Surveys have shown that such intake has not increased despite the noble encouragement of public service and government agencies (1). Some nutritional companies have chosen to invest resources into ascertaining whether extracts taken from fruits and vegetables could confer the same beneficial effects in humans without requiring individuals to alter lifestyle or eating habits.

Findings in previous research indicate that the elderly may especially benefit from increased levels of antioxidants (4). The risk of cancer and heart disease increases with age (5). Additionally, oxidative damage to cells has been proposed as a mechanism for the aging process (6,7,8). Oxidative insults to DNA can lead to mutations in crucial genes, which ultimately may lead to cancer (5,7, 9,10). Potent antioxidants and nutrients from fruits and vegetables may be able to quell the effects of oxidative DNA damage in the aged, in addition to lowering rates of heart disease and the overall risk for cancer (11,12, 13,14).

Measuring damage to DNA can be accomplished using several methods. The comet assay is a sensitive test that can detect small changes in the number of strand breaks (produced by endogenous or exogenous factors) within a nucleus (15). Given the ease of obtaining blood cells and the capacity of the comet assay to discern minute changes in cellular DNA, this appeared to be a suitable model for determining the effect of fruit and vegetable extracts on the level of DNA damage in seniors.

This initial study, therefore, was to determine whether a commercially available product containing fruit and vegetable extracts from a wide variety of sources could decrease the levels of DNA damage in the peripheral lymphocytes of a group of elderly subjects in lieu of changing the daily habits of the individuals.

MATERIALS AND METHODS

Subjects

20 subjects over the age of sixty were recruited for this 3-month study from a database of participants in previous research (this database is composed of approximately 200 healthy males and females over 60 years of age). Additional subjects were recruited using an advertisement in a retirement community newspaper. Subjects were screened for past medical history, alcohol consumption, and smoking status. Only subjects over 60 years old were eligible for participation. Active cancer or uncontrolled diabetes disqualified individuals from participating. Blood samples were taken from each subject prior to and after the supplementation period (80 days). The study was approved by the Human Subjects Committee of the University of Arizona (where the subjects were recruited and received supplementation) and all subjects provided written informed consent prior to their participation. During their second and third visits, subjects were given a supply of

supplements to last until their next visit. Subjects were instructed to consume 2 fruit capsules in the morning with an 8 oz glass of water and a meal and 2 vegetable capsules in the evening with an 8 oz glass of water and a meal. These directions were emphasized verbally and in writing at each visit. Subjects were otherwise instructed to eat as they normally would. Subjects were also instructed to bring any remaining pills to the clinic at day 40 and day 80, at which time pill counts were conducted.

Supplements

The fruit and vegetable extracts were provided by NSA International (Memphis, TN) and consisted of dried fruit and vegetable powders prepared as previously described (16). Briefly, fruit supplements contained 850 mg fruit powder per capsule and contained extracts from apples, oranges, pineapples, papaya, cranberries, and peaches. Vegetable supplements contained 750 mg vegetable powder per capsule and contained extracts from carrots, parsley, beets, broccoli, kale, cabbage, spinach, and tomatoes. The manufacturer claims that each capsule contains the nutrients of one to two of every fruit or vegetable mentioned. Supplements used were from the same respective lots.

Blood Sample Treatment

Blood samples were collected using sodium heparinized Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells were isolated from whole blood using density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) at 1900×g for 20 minutes. Cells were collected, washed twice with sterile PBS, and frozen in 8% DMSO at -70°C until Comet analysis could be performed.

Comet Assay

Frozen samples were thawed at 37°C and counted for viability using trypan blue exclusion. The comet assay was carried out as described previously (17). 20,000 cells from each sample were embedded in 0.75% low-melting point agarose (final concentration) and layered on custom frosted microscope slides (18). Cells were lysed, electrophoresed, and stained with propidium iodide for DNA visualization (figure 1). Each sample was assayed in triplicate under blind conditions, and 70 comets were analyzed from each slide. Computerized microscopic analysis of comets was carried out at 320x using a Zeiss epifluorescence microscope with attached CCD camera and GenII intensifier. Comet analysis software was provided by Dr. Peggy Olive (British Columbia Cancer Research Center).

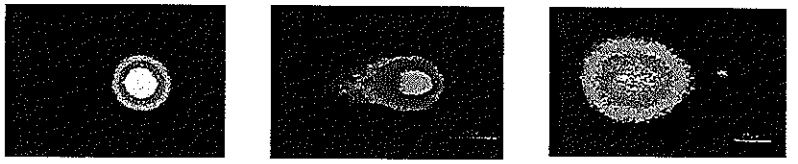

Figure 1– A typical control nucleus (left); a moderately damaged nucleus (middle); a heavily damaged nucleus (right). Each image is oriented with the nucleus to the right.

Determination of Carotenoids and Vitamin E in Serum

250 µl of ethanol (containing 0.1% BHT anti-oxidant) were added to a 250 µl aliquot of serum in order to precipitate proteins. After vortexing, analytes were extracted into hexane, evaporated under nitrogen, and then re-dissolved in mobile phase (solvent A— acetonitrile (CAN)- tetrahydrofuran (THF) (85:15, v/v), 250 ppm butylated hydroxytoluene (BHT), 0.05% triethylamine (TEA)). 50 µl of extractant were injected directly into the high-performance liquid chromatography (HPLC) system. Carotenoids and α-tocopherol were separated by a 5 µm ultrasphere ODS column (4.6 x 250mm; Beckman Instruments, San Ramon, CA) and detected at the wavelengths of 452 nm and 300 nm by the method of Xu et al. (19). The solvent system consisted of 95% solvent A and 5% solvent B (50 mM ammonium acetate in methanol with 0.05% TEA) and was delivered at a flow rate of 2.5 ml/minute. The retention times for lutein/zeaxanthin, β-cryptoxanthin, α-tocopherol, lycopene, α-carotene, and β-carotene were 1.94, 4.24, 4.83, 5.50, 9.45, and 10.13 minutes, respectively. The total run time for a single analysis of a sample was 13 minutes. Analytic quantification was performed by the external standard method. Extinction coefficients were used to spectrophotometrically validate the final solution concentrations. Standard reference material 968b (fat-soluble vitamins and cholesterol in human serum) supplied by the National Institute of Standards and Technology (NIST, Gathersberg, MD) was used for assigning values to in-house control materials.

RESULTS

Subjects reported that supplements were consumed as directed. Pill counts were conducted at the end of each month revealing that 99.99% of the fruit extract was consumed and 99.96% of the vegetable extract was consumed. Commonly, the subjects expressed an increased feeling of overall well-being and improved regularity.

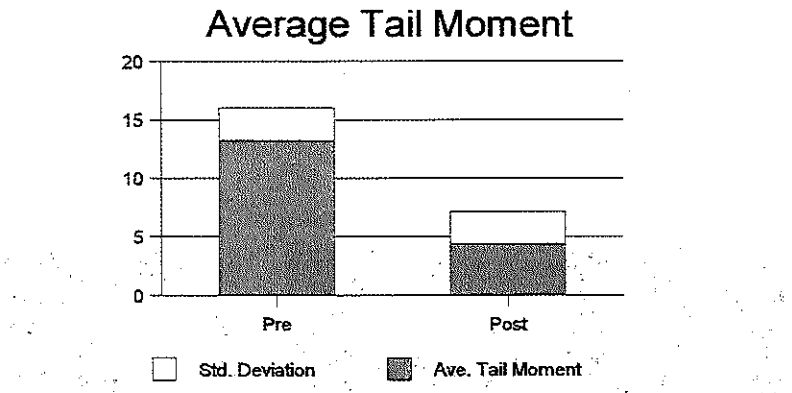

Figure 2-- Average reduction of DNA damage in an elderly study group. Post-intervention tail moment was significantly lower than pre-treatment measurements ($p<0.0001$).

Table I — Human Subjects Data

SUBJECT	DAMAGE (PRE)*	DAMAGE (POST)*	SEX	AGE ($\bar{x}$ = 68)	SMOKING STATUS
f21	10.29	2.58	F	86	N
f24	12.32	2.59	M	68	N
f26	11.75	3.52	M	67	N
f27	16.74	6.33	F	66	N
f28	10.24	6.74	F	65	N
f29	11.95	1.63	F	64	N
f30	18.23	1.61	F	64	N
f33	10.50	4.09	F	70	Y
f34	13.85	2.41	M	64	N
f35	11.12	5.33	F	60	N
f36	7.87	4.03	F	63	N
f42	16.59	4.28	M	65	N
f43	13.56	6.72	F	74	Y
f44	11.20	3.35	F	77	Y
f46	16.26	3.78	F	79	N
f48	12.70	4.22	M	62	Y
f49	15.40	3.64	M	72	N
f52	15.24	2.52	F	67	Y
f56	13.67	4.68	M	61	N
f57	15.78	14.24	F	68	Y

*Damage is given in tail moment (fraction of DNA in tail X length of tail).

Individual data for each participant in the study are given in table I. Pre- and post-treatment samples were compared using a paired t-test (MINITAB). Data obtained from comet assay analysis demonstrated a significant drop ($p<0.0001$) in the amount of observable DNA damage in the peripheral lymphocytes of test subjects following intervention with fruit and vegetable extracts. Average tail moment for pre- and post-treatment lymphocytes was 13.24 ± 2.77 and 4.41 ± 2.76 respectively (figure 2). Analysis of covariance failed to show a relationship between nuclear DNA damage and age ($p= 0.734$), sex ($p= 0.815$), or smoking status ($p= 0.355$) (figure 3 a, b, c; fig 3a is given as an average of subjects when there was more than one subject per age group). Although frozen for similar durations, viability of cells following recovery from the freezer was also higher for the treated samples (not shown).

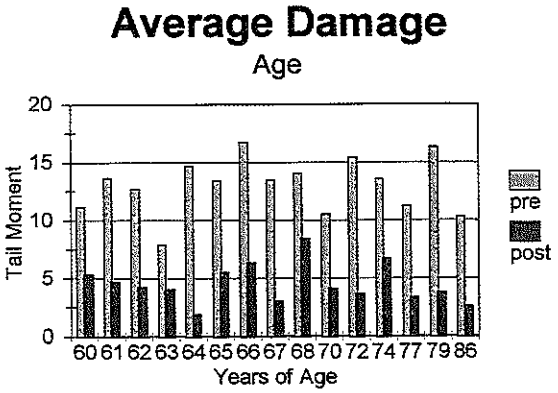

Figure 3a– DNA damage was not correlated with age in this study (p=0.734)

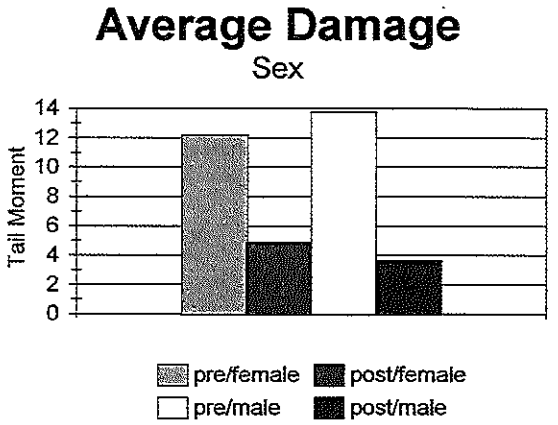

Figure 3b– Sex was not a significant factor in the level of DNA damage (p=0.815).

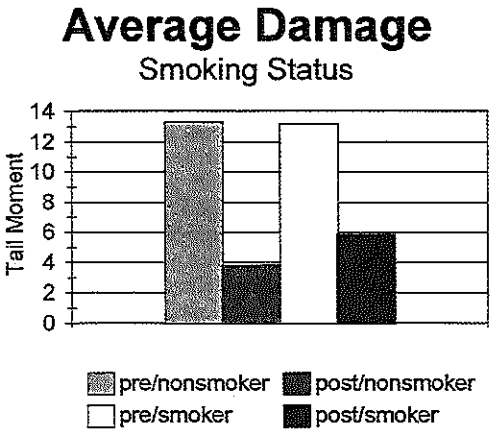

Figure 3c– Tests showed no significant difference in the responses of smokers and non-smokers (p=0.355)

Analysis of blood serum antioxidants following treatment showed a statistically significant increase in the levels of β-carotene (p<0.0001) and β-carotene (p= 0.0038) (Table II). Luten/zeaxanthine, lycopene, and α-tocopherol serum levels did not change significantly following intervention with fruit and vegetable extracts.

Table II–Serum Antioxidant Levels
(mean ± s.d.)

ANTIOXIDANT (μg/ml)	PRE	POST	p-VALUE
LUTEIN/ZEAXANTHIN	0.229±0.085	0.291±0.117	0.065
β-CRYPTOXANTHIN	0.090±0.052	0.089±0.047	0.934
LYCOPENE	0.308±0.151	0.346±0.139	0.416
α-CAROTENE	0.072±0.044	0.267±0.098	<0.0001
β-CAROTENE	0.377±0.043	0.842±0.052	0.0038
α-TOCOPHEROL	25.792±9.050	30.377±9.475	0.126

DISCUSSION

Consumption of fruits and vegetables has been demonstrated to be a compelling factor correlated with reduced risk for cancer (20, 21, 22). Other diseases can also be diminished by the actions of chemicals derived from produce (22). A companion study to our research has also shown fruit extracts to enhance immune function, specifically Natural Killer cell activity, T cell mitogenesis, and B cell mitogenesis increased (personal communication— Ronald Watson; see also ref 22).

Given the health advantages of fruit and vegetable consumption, it would not seem difficult to persuade the public to increase their intake of produce. Unfortunately, this is not the case. Consumption of fruits and vegetables has not risen notwithstanding coaxing from various health organizations (23). Though not a replacement for a healthy diet and lifestyle, finding an alternative method to elicit similar effects as fruits and vegetables may therefore be a worthwhile cause. Commercial products such as JuicePlus™ are intended to fill that role.

Each type of fruit or vegetable has an individualized "fingerprint" of beneficial compounds. High levels of specific chemicals in one fruit can be augmented through dietary combination with other fruits or vegetables. For example, apples are high in the flavonoid antioxidant quercetin yet are lower in vitamin C and β-carotene (20). Combining citrus fruits and carrots with apples furnishes a broader range of antioxidants (and additional phytonutrients) than apples alone. The selection of a variety of produce from which extracts are made provides a greater spectrum of beneficial compounds, perhaps more than an average person could get eating raw produce. Some of the chemicals found in the extracts used in this study include: quercetin and vitamin E (apples, cabbage, kale) (24), vitamin C (citrus fruits) (25), sulforaphane (broccoli) (26), flavonoids (cranberries) (27), β-carotene (carrots) (25), and lycopene (tomatoes) (28, 29, 30).

Following intervention with these fruit and vegetable extracts, the amount of DNA damage found in the peripheral lymphocytes of test subjects was substantially reduced. Further, the beneficial effects did not appear to follow indicators like age, sex, and smoking status. Although the response to treatment was not uniform, it seemed to be helpful for the majority of the test group (subject f57 was the only notable exception) without regard to the aforementioned factors. Such a broad range of action may underscore the potential usefulness of these extracts in the elderly, although these results must be regarded as tentative and only mildly inferential until further scientific evidence becomes available.

Intake of fruits and vegetables is beneficial for people of all ages, however seniors are at greater risk for DNA damage than younger people. Work by Ames and colleagues have shown that the level of metabolism is seven times higher in elderly rats than in adolescents, resulting in twice the number of DNA lesions (5). This may be due in part to "leakage" from the mitochondrial electron transport chain as an individual ages (5, 8). Regardless of the cause, higher amounts of DNA damage can lead to mutations. When sufficient mutations accumulate in specific genes, cells can become transformed and develop into tumors (4). Supplementation with fruit and vegetable extracts is intended to diminish the likelihood of this occurrence. Whether this line of reasoning is feasible is not fully established because epidemiological studies must account for many more factors than just oxidation or DNA damage. However, the manifest decrease in DNA damage in this initial study is encouraging.

A possible explanation for the reduction of the DNA damage in the lymphocytes of test subjects may be found in the serum levels of the carotenes (both α and β). Carotenes significantly increased following the course of intervention. Recent studies suggest otherwise, having shown similar increases in serum carotenoids but finding that these levels were more indicative of fruit and vegetable intake than a mechanism for reducing oxidative DNA damage in human lymphocytes (31, 32). Other compounds may also have been involved, but no data acquired from the tests performed support such a position. The increase in lymphocyte activity as mentioned above may also have bearing on the improved state of the cells tested.

Aside from the potential value of fruit and vegetable extracts in the elderly, this study displays the utility of the comet assay in evaluating the effects of compounds in humans using minimally invasive techniques. This test can plainly be used to assess trends in populations (33, 34, 35). A great advantage of the comet assay is the use of single nuclei for measurements– demonstrating the impact of treatments at the cellular and subcellular (genomic) level.

Aspects not addressed by this study include the amounts of each compound in specified fruits and vegetables and therefore in the extracts. Since these levels are modulated by the environment in which the plants are grown and the overall well-being of the producing plants, strict controls of manufacture are necessary to maintain product consistency.

This study suggests that supplementation with fruit and vegetable extracts may decrease the amount of DNA damage in a population of elderly subjects. Indeed, it exhibits positive results without apparent influences from age, sex, or smoking status. However, the size of the sample and the lack of a placebo control group limits the number and strength of conclusions that can be drawn from the data. A large scale study incorporating double blind conditions with placebo controls would greatly expand the latitude in which researchers can make conclusions. This research spanned a relatively short treatment period and the results were favorable, therefore we believe further investigation into long-term effects may be warranted.

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